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Sodium Cyanide Increases Cytosolic Free Calcium: Evidence for Activation of the Reversed Mode of the Na^+/Ca^{2+} Exchanger and Ca^{2+} Mobilization from Inositol Trisphosphate-Insensitive Pools[1]

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Cyanide has been shown to affect calcium homeostasis. Cyanide causes an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells (Johnson *et al.*, 1987; Maduh *et al.*, 1990), rabbit carotid body chemoreceptors (Biscoe and Duchen, 1989; Sato *et al.*, 1991), rat ventricular myocytes (Eisner *et al.*, 1989), rat osteoclasts (Teti *et al.*, 1989), and *Leishmania donovani* promastigotes (Philosoph and Zilberstein, 1989). In PC12 cells the increase is believed to be activated by voltage-gated Ca^{2+} channels (Johnson *et al.*, 1987). In rabbit carotid body chemoreceptors it is due to inhibition of Na^+/Ca^{2+} exchange (Biscoe and Duchen, 1989; Sato *et al.*, 1991). In *Leishmania donovani* promastigotes, it results from a Ca^{2+} mobilization from intracellular Ca^{2+} pools (Philosoph and Zilberstein, 1989).

It is believed that an abrupt increase in $[Ca^{2+}]_i$ is associated with cell death (Farber, 1981; Orrenius *et al.*, 1989; Trump *et al.*, 1989) mediated by activation of Ca^{2+}-dependent proteases (Geeraerts *et al.*, 1991). However, Snyder *et al.* (1993) reported that the cyanide killing of hepatocytes correlated closely with changes in the mitochondrial membrane potential. On the contrary, Sakaida *et al.* (1992) concluded that cyanide kills cultured hepatocytes because of an alteration in the interaction between the cytoskeleton and the plasma membrane.

The relationship between cyanide and human epithelial cells has not previously been described. Because cyanide is readily absorbed from all routes, including the skin, and death can occur within minutes or hours depending on the route of exposure (Rumack and Peterson, 1980), human skin epidermoid A-431 cells were used to examine the effects of cyanide on $[Ca^{2+}]_i$, to investigate the mechanisms underlying the increase in $[Ca^{2+}]_i$ that occurred following cyanide exposure and to study the relationship between the increase in $[Ca^{2+}]_i$ and cell viability after cyanide exposure. The pathways involved in Ca^{2+} homeostasis in these cells have been thoroughly characterized and are not different from other types of cells (Galloway *et al.*, 1990; Lin *et al.*, 1992). In this study we found that cyanide increased $[Ca^{2+}]_i$ and decreased inositol trisphosphates ($InsP_3$). The $[Ca^{2+}]_i$ increase was because of a Na^+ influx that activated the re-

[1] Views presented in this paper are those of the authors; no endorsement by the Department of the Army or the Department of Defense has been given or should be inferred.

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versed mode of the Na^+/Ca^{2+} exchanger, thereby leading to an increased Ca^{2+} entry. The Ca^{2+} influx induced, in turn, a Ca^{2+}-stimulated mobilization of Ca^{2+} from intracellular Ca^{2+} pools through ryanodine receptor channels that we found were sensitive to ionomycin but not to bradykinin or monensin. This mechanism underlying the $[Ca^{2+}]_i$ increase caused by NaCN was different from those mechanisms found in other types of cells (Biscoe and Duchen, 1989; Johnson *et al.*, 1987; Philosoph and Zilberstein, 1989; Sato *et al.*, 1991), but was similar to the heat shock-induced increase in $[Ca^{2+}]_i$ (Kiang *et al.*, 1992).

MATERIALS AND METHODS

Cell culture. Human epidermoid carcinoma A-431 cells (American Type Culture Collection, Rockville, MD) were grown on glass coverslips (9 × 35 mm, Clay Adams, Lincoln Park, NJ) incubated at 37°C in a 5% CO_2 atmosphere. The tissue culture medium was Dulbecco's modified eagle medium supplemented with 0.03% glutamine, 4.5 g/liter glucose, 25 mM Hepes, 10% fetal bovine serum, 50 μg/ml penicillin, and 50 U/ml streptomycin (GIBCO/BRL, Gaithersburg, MD). Cells were fed every 3–4 days (Giard *et al.*, 1973). Cells from passages 28–50 were used for experiments.

Measurements of cytosolic free Na^+ and Ca^{2+}. Confluent monolayers of cells were loaded with 5 μM SBFI-AM plus 0.2% pluronic F-127 (to make cells more permeable to the probe) for 2 hr at 37°C before measurements of cytosolic free Na^+ ($[Na^+]_i$) or with 5 μM fura-2AM plus 0.2% pluronic F-127 for 60 min for measurements of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). Pluronic F-127 at 0.2% does not affect the relationship between fluorescence ratio and actual $[Ca^{2+}]_i$ because the $[Ca^{2+}]_i$ of cells were similar after being incubated in the dye buffer with or without pluronic F-127 (data not shown). Cells were then washed with Na^+ Hanks' solution twice before fluorescence measurements. The fluorescence signal was measured with a PTI DeltaScan spectrofluorometer (Photon Technology International, Inc., South Brunswick, NJ). The rate of leakage of fura-2 from the cells and the method to determine $[Ca^{2+}]_i$ have been published previously (Grynkiewicz *et al.*, 1985; Gunter *et al.*, 1988; Kiang, 1991; Kiang *et al.*, 1992; Kiang and McClain, 1993).

To determine $[Na^+]_i$, the fluorescence signal was measured with emission at 510 nm and dual excitation at 340 and 385 nm (slit width 4 nm). Autofluorescence from cells not loaded with dye was in the range of 3.5–8.5 × 10^3 photons/sec and was subtracted from the SBFI signal (after subtraction, bound form: 1.2–1.4 × 10^5 photons/sec; free form: 1.5–2.0 × 10^4 photons/sec). SBFI leaked out of A-431 cells at a rate of 0.11 ± 0.07%/min (n = 7). Cells were washed thoroughly in Hanks' solution within 10 sec before they were transferred to a cuvette to measure $[Na^+]_i$. Because the apparent affinity of SBFI for Na^+ is reported to be sensitive to pH (Kawanishi *et al.*, 1991), the fluorescence signal at different pHs was determined using a method described by Harootunian *et al.* (1989). Calibrated Na^+ solutions used to determine $[Na^+]_i$ were prepared from appropriate mixtures of high Na^+ and high K^+ solutions. The high Na^+ solution was identical to regular Hanks' solution, and the high K^+ solution differed by having Na^+ completely replaced by K^+.

Ca^{2+} efflux. To measure Ca^{2+} efflux, cells were incubated in a solution containing $^{45}Ca^{2+}$ (final concentration 1 μCi/ml, 0.3 mCi/mmol) at 37°C for 1 hr. The total $^{45}Ca^{2+}$ radioactivity incorporated into cells was counted before and after 10 min of NaCN treatment.

Inositol trisphosphate measurements. Cells were grown on 6-well tissue culture plates (2 × 10^6 cells/well) and incubated with [^{3}H]myoinositol (2 μCi/ml, 0.22 nmol/ml) in growth medium for 24 hr. The cells were washed with Na^+ Hanks' solution twice before the NaCN treatment. The reaction was stopped by the addition of 3 ml of ice-cold 4.5% $HClO_4$:Na^+ Hanks' solution (2:1, v/v) to each well. The plate was chilled for 30 min and cells were removed by scraping. The supernatants were prepared for isolation of the [^{3}H]inositol metabolites by adjusting the pH to 8.0 with a solution of 0.5 M KOH, 9.0 mM $Na_2(BO_4)_2$, and 1.9 mM EDTA. The samples were stored at −20°C overnight. After thawing, the $KClO_4$ salts precipitated and were removed by centrifugation at 2500 rpm for 10 min. One hundred microliters of the supernatant was used to determine the total radioactivity in the sample. The remainder was applied to 1 ml of suspended Dowex AG 1-X8 resin in the formate form (100–200 mesh). The [^{3}H]inositol phosphates were eluted according to the method of Berridge and Irvine (1989).

TABLE 1
Fura-2 and SBFI Release by Digitonin or Triton X-100

Treatment	Concentration	Remaining fluorescence (%) Fura-2	SBFI
Digitonin	20 μM	11.3 ± 2.7	17.3 ± 2.2
	100 μM	5.4 ± 0.4	12.0 ± 1.2
Triton X-100	0.2%	0.3 ± 0.2	2.0 ± 0.9

Note. Cells were loaded with the fluorescence probe and the results were calculated as the percentage of fluorescence remaining after exposure to the detergents (n = 3).

Solutions. Hanks' solution contained: 145 mM NaCl, 4.6 mM KCl, 1.3 mM $MgCl_2$, 1.6 mM $CaCl_2$, and 10 mM Hepes (pH 7.40 at 24°C). In Na^+-free Hanks' solution, *N*-methyl-(+)glucamine (NMG) was used to substitute for equimolar concentrations of Na^+. Ca^{2+}-free Hanks' solution was prepared by adding 10 mM EGTA to nominally Ca^{2+}-free Hanks' solution.

Statistical analysis. All data are expressed as the means ± SEM. Analysis of variance, Student's t test, Studentized range test, and Bonferroni's inequality were used for comparison of groups (Sokal and Rohlf, 1969). Curve fitting was determined using the Inplot program (GraphPad, San Diego, CA).

Chemicals. Fura-2AM, SBFI-AM, nigericin, ionomycin, and 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) were purchased from Molecular Probes, Inc. (Eugene, OR). Other chemicals used in this study were gramicidin D, monensin, amiloride, bovine serum albumin, *N*-methyl-(+)glucamine, verapamil hydrochloride, $LaCl_3$, $CdCl_2$, ouabain, veratridine, tetrodotoxin, ryanodine, ruthenium red, monensin, caffeine (Sigma Chemical Co., St. Louis, MO), nifedipine (Calbiochem, Torrance, CA), 5-(*N*,*N*-hexamethylene)amiloride (Research Biochemicals Inc., Natick, MA), and $^{45}Ca^{2+}$ (ICN, Irvine, CA). 1-{6-[(17β-3-methoxyestra-1,3,5(10)-triene-17-yl)amino]hexyl}-2,5-dione (U-73122) was generously provided by The Upjohn Co. (Kalamazoo, MI). Dichlorobenzamil was provided by Dr. Peter K. S. Siegl (Merck & Co, Inc., West Point, PA).

RESULTS

Since compartmentalization of fura-2 and SBFI had not been previously determined in A-431 cells, we identified the subcellular location of these probes after sequential treatment with 20 μM digitonin, 100 μM digitonin, and 0.2% Triton X-100, with open cellular compartments corresponding to cytosol, nonmitochondrial organelles, and mitochondria, respectively (Gores *et al.*, 1989; Kawanishi *et al.*, 1991; Nieminen *et al.*, 1990). Table 1 shows that

these probes were loaded principally into cytosol (83–89%), which confirms their usefulness for measuring $[Ca^{2+}]_i$ and $[Na^+]_i$.

NaCN-Induced Increase in $[Ca^{2+}]_i$

The resting $[Ca^{2+}]_i$ in adherent cells at 37°C in a normal Na^+ Hanks' solution was 85 ± 8 nM (n = 141). In the absence of external Ca^{2+}, the resting $[Ca^{2+}]_i$ decreased to 59 ± 7 nM (n = 7; $p < 0.05$). These data indicate that there is a considerable contribution of external Ca^{2+} to the resting $[Ca^{2+}]_i$ (Kiang, 1991; Kiang *et al.*, 1992). Cells exposed to NaCN exhibited an increase in $[Ca^{2+}]_i$ that was time- and cyanide concentration-dependent (Figs. 1A and 1B). Trypan blue dye exclusion and replating efficiency were used to measure the viability of NaCN-treated cells. Adherent cells treated with 10 mM NaCN for 10 min maintained their ability to exclude trypan blue over a period of 60 min. The replating efficiency of these cells 1 day after NaCN treatment was not different from untreated cells (control, 91 ± 2%; NaCN, 92 ± 2%, n = 6 for both groups, $p > 0.05$).

We observed that $[Ca^{2+}]_i$ returned to the baseline 45 min after cells were exposed to NaCN (10 mM, 10 min) (Fig. 1C). These cells remained viable after NaCN was removed (data not shown). All of the remaining mechanistic studies employed a 10-min exposure of the cells to 10 mM NaCN because this treatment regimen was both nonlethal and produced a sizeable increase in $[Ca^{2+}]_i$ of approximately 250%. Each experiment was performed with its own controls because the resting $[Ca^{2+}]_i$ was different in different experiments.

Effect of Polyvalent Ions on the NaCN-Induced Increase in $[Ca^{2+}]_i$

In an effort to determine the source of the NaCN-induced increase in $[Ca^{2+}]_i$, a series of experiments were performed in the absence of extracellular Ca^{2+}. As Fig. 2 depicts, removal of extracellular Ca^{2+} prevented the NaCN response. When Ca^{2+} was added to the buffer, an increase in $[Ca^{2+}]_i$ by NaCN was observed, suggesting that this response is due primarily to Ca^{2+} influx. The influx of Ca^{2+} into other kinds of cells can be blocked by Cd^{2+} or La^{3+} (inorganic Ca^{2+} channel blockers) in the external medium (Hagiwara, 1983; Trosper and Philipson, 1983). In A-431 cells both Cd^{2+} and La^{3+} inhibited the increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 3), with La^{3+} being more effective than Cd^{2+}. The $K_{1/2}$ for La^{3+} and Cd^{2+} were 0.2 and 28 μM, respectively, similar to the values determined in synaptosomes (Nachshen, 1984) and cardiac sarcolemmal vesicles (Trosper and Philipson, 1983).

Agents such as verapamil and nifedipine that are known to interfere with L-type voltage-gated Ca^{2+} channels (Hagiwara, 1983) were also tested. Neither agent up to 1 mM inhibited the NaCN response (Table 2). These results ruled out the possibility that Ca^{2+} influx occurred through L-type voltage-gated channels.

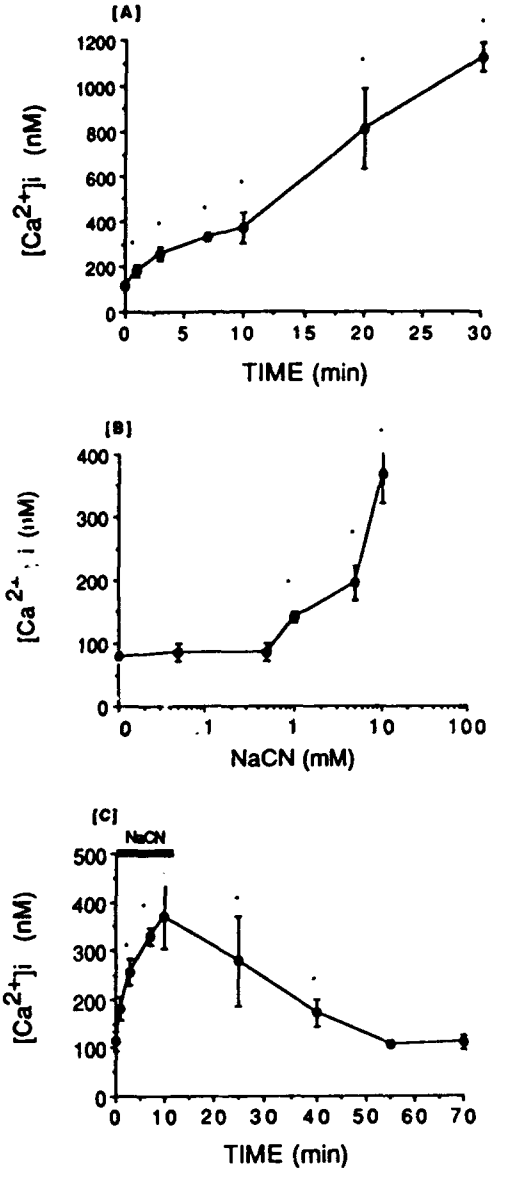

FIG. 1. (A) Increases in $[Ca^{2+}]_i$ induced by different durations of exposure to NaCN. Cells were treated with 10 mM NaCN for various periods of time before $[Ca^{2+}]_i$ measurements (n = 3–5). (B) Increases in $[Ca^{2+}]_i$ induced by various concentrations of NaCN. Cells were treated with NaCN (10 mM) for 10 min (n = 3). (C) Recovery of $[Ca^{2+}]_i$ levels after the NaCN-induced increase in $[Ca^{2+}]_i$. Cells were exposed to NaCN for 10 min, washed thoroughly, and then $[Ca^{2+}]_i$ was monitored at intervals up to 70 min (n = 3). *$p < 0.05$ vs 0 min or controls.

Ca^{2+} efflux was probably not affected by NaCN because Ca^{2+} retention in cells treated with 10 mM NaCN for 10 min was not statistically different from that in untreated cells (control: 8.3 ± 0.5 nmol/mg protein; NaCN: 7.5 ± 0.4 nmol/mg protein, n = 3, $p > 0.05$, Student's t test). This

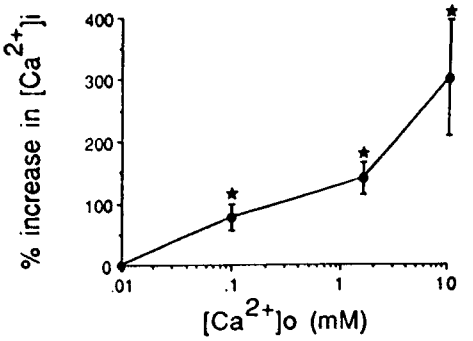

FIG. 2. Dependence of NaCN-induced increase in $[Ca^{2+}]_i$ on external $[Ca^{2+}]$. Cells were exposed to NaCN for 10 min in Na^+ Hanks' solution containing different Ca^{2+} concentrations ($[Ca^{2+}]_o$) (n = 3). *p < 0.05 vs 0.01 mM.

indicates that the increase in $[Ca^{2+}]_i$ was probably not due to an impairment of Ca^{2+} efflux.

Effect of Extracellular Na^+ on the NaCN-Induced Increase in $[Ca^{2+}]_i$ and $[Na^+]_i$

Because La^{3+} can, in addition to its action as a Ca^{2+} channel blocker, also inhibit Na^+/Ca^{2+} exchange (Kaczorowski *et al.*, 1984; Trosper and Philipson, 1983), it remained to be determined whether the La^{3+} effect might also indicate a role for Na^+/Ca^{2+} exchange in the NaCN-induced increase in $[Ca^{2+}]_i$. A-431 cells have been shown to possess a Na^+/Ca^{2+} exchanger (Kiang *et al.*, 1992). If this exchanger is activated when cells are exposed to NaCN, then we *might* observe changes in intracellular Na^+ concentration ($[Na^+]_i$) after NaCN treatment. We determined that NaCN does alter $[Na^+]_i$. After treating cells with NaCN (10 mM, 10 min), the intracellular Na^+ concentration ($[Na^+]_i$) was 16 ± 1 mM (n = 4), which differed from levels measured in control cells (6.2 ± 1.5 mM, n = 9). The removal of extracellular Na^+ inhibited the NaCN-induced increase in $[Na^+]_i$, which suggests that the increase in $[Na^+]_i$ is because of Na^+ entry.

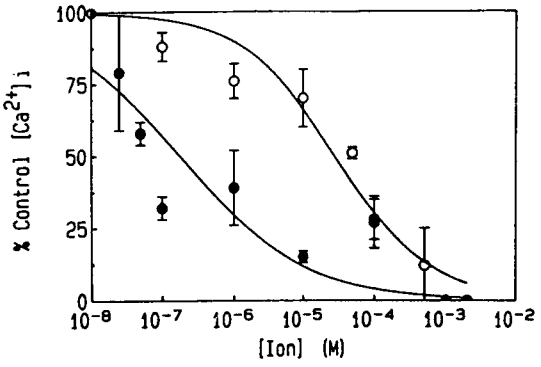

FIG. 3. Inhibition by La^{3+} and Cd^{2+} of the NaCN-induced increase in $[Ca^{2+}]_i$. Cells were treated with NaCN (10 mM, 10 min) in the presence of various concentrations of La^{3+} (●) or Cd^{2+} (○) (n = 3), and two sigmoid-curved fits are presented. The calculated $K_{1/2}$ for La^{3+} and Cd^{2+} are 0.2 and 28 μM, respectively.

TABLE 2

Voltage-Gated Ca^{2+} Channel Blockers or Phospholipase C Antagonist Did Not Prevent the NaCN-Induced Increase in $[Ca^{2+}]_i$

		$[Ca^{2+}]_i$	
Treatment	Concentration	Control	NaCN
Vehicle	—	82 ± 9	250 ± 22*
Verapamil	1 mM	91 ± 19	266 ± 83*
Nifedipine	1 mM	93 ± 18	350 ± 60*
U-73122	5 μM	115 ± 18	246 ± 56*

Note. Cells were treated with 10 mM NaCN for 10 min (n = 3).
* p < 0.05 vs control.

In order to understand more of the role of Na^+ we performed a series of experiments to modify Na^+ gradients across the plasma membrane by changing extracellular Na^+ or $[Na^+]_i$ and measuring their effect on the NaCN-induced $[Ca^{2+}]_i$ increase. We increased $[Na^+]_i$ by incubating cells in Na^+ Hanks' solution containing 1 mM ouabain (a Na^+/K^+-ATPase blocker) for 30 min at 37°C. The $[Na^+]_i$ in these cells increased to 26 ± 4 mM (n = 3). The $[Ca^{2+}]_i$ was stable but higher than that in cells not exposed to ouabain (control: 58 ± 9 nM, ouabain: 111 ± 17 nM, n = 3–4, p < 0.05), which served to confirm the presence of a Na^+/Ca^{2+} exchanger in these cells. NaCN increased $[Ca^{2+}]_i$ and $[Na^+]_i$ in ouabain-treated cells by 241 ± 56% (n = 3) and 238 ± 10% (n = 3), respectively. These increases were less (p < 0.05) than those observed in cells not treated with ouabain, where $[Ca^{2+}]_i$ and $[Na^+]_i$ increased by 367 ± 15 and 267 ± 16% (n = 3), respectively.

The removal of external Na^+ inhibited the $[Ca^{2+}]_i$ increase. The NaCN-induced increase in $[Ca^{2+}]_i$ was induced by buffer concentrations of Na^+ greater than 30 mM and was maximal in the presence of greater than 110 mM external Na^+ (Fig. 4). The $K_{1/2}$ for extracellular Na^+ was 85 mM.

The contribution of Na^+ from 10 mM NaCN was included when calculating the final concentration of external Na^+ in these experiments, but the quantity of Na^+ derived from the 10 mM NaCN did not affect the cyanide-induced increase in $[Ca^{2+}]_i$. This is because all studies (unless indicated otherwise) were conducted in Hanks' solution containing 145 mM Na^+, a concentration above the 110 mM that stimulated a maximal increase in $[Ca^{2+}]_i$. The results of these experiments clearly show that the NaCN-induced increases in $[Ca^{2+}]_i$ depend on $[Na^+]_i$.

Effect of Amiloride and Dichlorobenzamil on the NaCN-Induced Increase in $[Ca^{2+}]_i$

The role of Na^+/Ca^{2+} exchange in the NaCN-induced increase in $[Ca^{2+}]_i$ can be tested by incubating cells with drugs that inhibit the exchanger. Amiloride and dichlorobenzamil, both of which have been shown to inhibit the

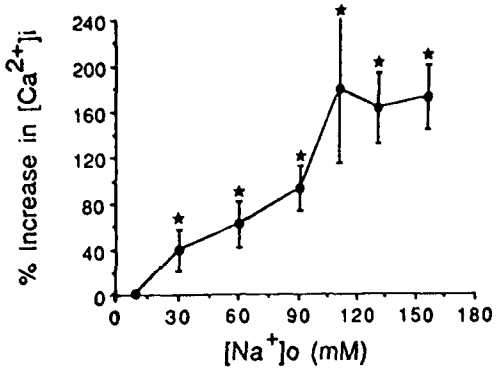

FIG. 4. Dependence of NaCN-induced increase in $[Ca^{2+}]_i$ on external $[Na^+]$. Cells were exposed to NaCN (10 mM, 10 min) in Hanks' solution containing different $[Na^+]$ (n = 3–5). *$p < 0.05$ vs data found with $[Na^+]_o$ = 10 mM.

Na^+/Ca^{2+} exchanger (Kiang *et al.*, 1992; Kleyman and Cragoe, 1988; Siegl *et al.*, 1984), blocked the increase in $[Ca^{2+}]_i$ induced by NaCN (Fig. 5). Cells were incubated in Na^+ Hanks' solution containing different concentrations of amiloride with or without NaCN. Cells were then washed thoroughly before $[Ca^{2+}]_i$ measurements. Amiloride at a concentration less than 1 μM had no effect on the resting $[Ca^{2+}]_i$. At 1 mM amiloride, there was a complete inhibition, with a $K_{1/2}$ of 5.4 μM. Dichlorobenzamil was 19 times more potent, with a $K_{1/2}$ of 0.28 μM. An inactive analog of amiloride for the Na^+/Ca^{2+} exchanger, 5-(*N,N*-hexamethylene)amiloride, used at concentrations up to 5 mM, did not block the NaCN effect, suggesting the inhibition produced by amiloride or dichlorobenzamil was specific.

It has been reported that the Na^+ channel also plays a role in intracellular Na^+ homeostasis and that amiloride and dichlorobenzamil inhibit Na^+ channels (Kleyman and Cragoe, 1988; Siegl *et al.*, 1984). Therefore, experiments were conducted with an agonist and antagonist of this channel. When cells were treated with the Na^+ channel agonist veratridine (1 mM), $[Na^+]_i$ increased from 6.2 ± 1.5 to 9.1 ± 2.0 mM (n = 4). Subsequent NaCN treatment elicited an increase in $[Ca^{2+}]_i$ of 215 ± 20% (n = 3), a level significantly lower than that observed after treatment with NaCN in the absence of veratridine (336 ± 15%, n = 4). The Na^+ channel antagonist tetrodotoxin at 5 μM did not inhibit the NaCN-induced increase in $[Ca^{2+}]_i$. At 50 μM tetrodotoxin, NaCN increased $[Ca^{2+}]_i$ only 30 ± 5% (n = 3, $p < 0.05$); at 100 μM, tetrodotoxin inhibited the NaCN effect completely. The data indicate that the Na^+ influx through tetrodotoxin-sensitive Na^+ channels contributes to the NaCN effect on $[Ca^{2+}]_i$.

Effect of TMB-8 and Ryanodine on the NaCN-Induced Increase in $[Ca^{2+}]_i$

It is known that the release of Ca^{2+} from intracellular pools can be stimulated by Ca^{2+} in certain types of cells (Kiang *et al.*, 1992; Kiang and McClain, 1993; Randriamampita *et al.*, 1991). If Ca^{2+} influx induces Ca^{2+} release from intracellular pools, an incubation with Ca^{2+} mobilization blockers should attenuate the total increase in $[Ca^{2+}]_i$ caused by NaCN. NaCN (10 mM, 10 min) increased $[Ca^{2+}]_i$ by only 69 ± 11 and 146 ± 48% (n = 3, $p < 0.05$), respectively, in cells treated with TMB-8 (100 μM) or ryanodine (100 μM). Because ryanodine receptor channels are known to be stimulated by Ca^{2+} and inhibited by ruthenium red (see review, Tsien, 1990), we tested the effect of treating A-431 cells with ruthenium red (10 μg/ml) for 10 min prior to treatment with NaCN. NaCN increased $[Ca^{2+}]_i$ only 56 ± 14% (n = 3, $p < 0.05$), suggesting that ryanodine receptor channels are involved. These results indicate that the NaCN-induced increase in $[Ca^{2+}]_i$ results from both Ca^{2+} influx and Ca^{2+} released from intracellular Ca^{2+} pools that have ryanodine receptors.

Effect of NaCN on Intracellular Ca^{2+} Pools

This laboratory (Kiang *et al.*, 1992) previously reported that A-431 cells have an intracellular Ca^{2+} pool that is sensitive to $InsP_3$ and can be depleted by bradykinin (Wheeler *et al.*, 1990). We, therefore, performed experiments to determine whether $InsP_3$ is involved in the increase in $[Ca^{2+}]_i$ in cells treated with NaCN. NaCN itself caused a decrease in $InsP_3$ that depended on both the duration of cyanide treatment and its concentration (Figs. 6A and 6B). Moreover, U-73122, which inhibits $InsP_3$ production (Kiang and McClain, 1993; Smallridge *et al.*, 1992; Smith *et al.*, 1990), failed to block the increase in $[Ca^{2+}]_i$ caused by NaCN when used at concentrations up to 5 μM (Table 2). These results suggest that Ca^{2+} released from intracellular pools caused by NaCN was not derived from the $InsP_3$-sensitive pool.

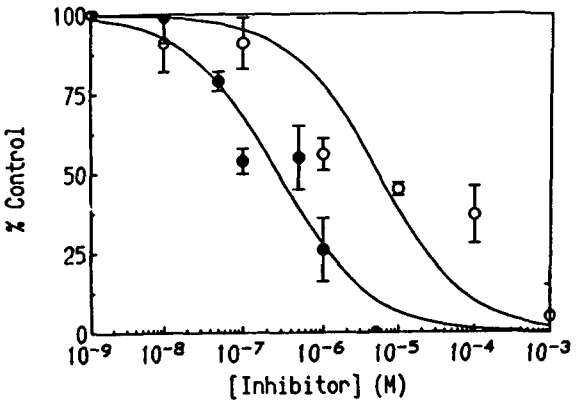

FIG. 5. Inhibition of the NaCN-induced $[Ca^{2+}]_i$ by amiloride (○) and dichlorobenzamil (●). Cells were exposed to NaCN (10 mM, 10 min) in Na^+ Hanks' solution containing different concentrations of either inhibitor. Cells were then washed thoroughly before $[Ca^{2+}]_i$ measurements. Two sigmoid-curved fits are presented. The calculated $K_{1/2}$ for amiloride and dichlorobenzamil are 5.4 and 0.28 μM, respectively.

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In A-431 cells, bradykinin (10 μM), monensin (200 μM), and ionomycin (1 μM) can deplete their respective intracellular Ca^{2+} pools (J. G. Kiang, unpublished data). The bradykinin-induced Ca^{2+} mobilization is mediated by $InsP_3$. To determine which non-$InsP_3$ sensitive pools responded to NaCN, cells were pretreated with bradykinin, monensin, and ionomycin. Table 3 shows the changes in $[Ca^{2+}]_i$ observed in cells treated with ionomycin, monensin, or bradykinin in the presence of 1.6 mM external Ca^{2+} prior to NaCN exposure. When NaCN was applied to cells treated with 1 μM ionomycin, a concentration that depleted the ionomycin-sensitive intracellular Ca^{2+} pool, the magnitude of the $[Ca^{2+}]_i$ increase was no greater than that which was attributable to Ca^{2+} influx alone (Table 3 and Fig. 7A). At 10 μM ionomycin, a concentration that saturated cells with Ca^{2+}, NaCN did not produce further changes in $[Ca^{2+}]_i$ (Fig. 7B). Both monensin and bradykinin at concentrations that depleted their respective Ca^{2+} pools did not have an effect on the NaCN-induced $[Ca^{2+}]_i$ increase (Figs. 7C and 7D, Table 3), indicating that $InsP_3$- and monensin-sensitive pools are not involved.

It is generally assumed that cellular mitochondria normally contain stores of Ca^{2+} that can be released as free Ca^{2+} after mitochondrial depolarization. Kawanishi *et al.* (1991) reported that mitochondrial Ca^{2+} uptake or release did not contribute to Ca^{2+} homeostasis in hepatocytes treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 100 μM). CCCP increased the resting $[Ca^{2+}]_i$ in A-431 cells from 171 ± 11 nM (n = 3) to 408 ± 53 nM (n = 3, $p < 0.05$). Treatment of these cells with 10 mM NaCN for 10 min still increased $[Ca^{2+}]_i$ by 334 ± 72% (n = 3, $p < 0.05$), suggesting that the NaCN-induced increase in $[Ca^{2+}]_i$ is not associated with a release of Ca^{2+} from mitochondria.

TABLE 3
Effect of Ionomycin, Monensin, and Bradykinin on the NaCN-Induced Increase in $[Ca^{2+}]_i$

Treatment	Concentration	Increase in $[Ca^{2+}]_i$ (nM)
NaCN alone		258 ± 67
Ionomycin	1 μM	133 ± 34*
	10 μM	0
Monensin	200 μM	248 ± 69
Bradykinin	10 μM	402 ± 100

Note. Cells were treated with NaCN (10 mM, 10 min) in the presence of ionomycin, monensin, or bradykinin (n = 3).
* $p < 0.05$ vs control cells, Student t test.

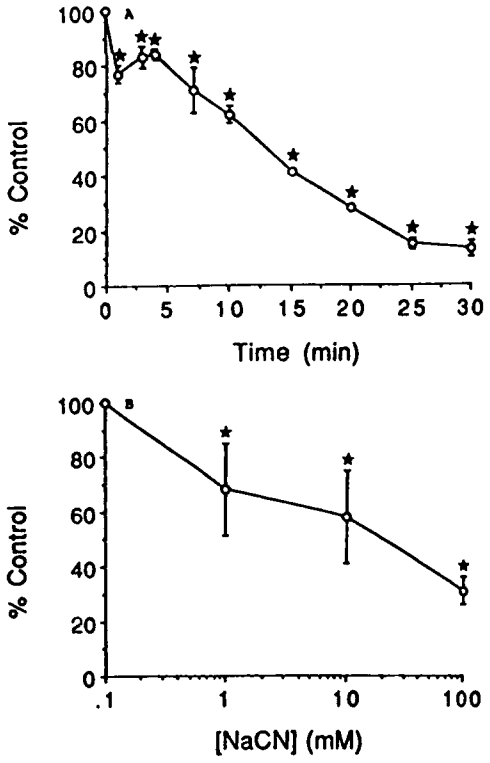

FIG. 6. Inhibition of inositol trisphosphate ($InsP_3$) production by NaCN. (A) $InsP_3$ production after exposing cells to 10 mM NaCN for various periods of time. (B) $InsP_3$ production after exposing cells to different concentrations of NaCN for 10 min (n = 3). *$p < 0.05$ vs 100%.

DISCUSSION

This study shows that NaCN induced a gradual increase in $[Ca^{2+}]_i$ in A-431 cells. The phenomenon is similar to the effect found in rat PC12 cells (Johnson *et al.*, 1987; Maduh *et al.*, 1990), rabbit carotid body chemoreceptors (Biscoe and Duchen, 1989), rat ventricular myocytes (Eisner *et al.*, 1989), and rat osteoclasts (Teti *et al.*, 1989). In A-431 cells the gradual increase is primarily due to an increase in $[Na^+]_i$ followed by a Ca^{2+} influx, because removal of extracellular Na^+ or Ca^{2+} prevented the cyanide-induced increase in $[Ca^{2+}]_i$. A reduction in Ca^{2+} efflux and a consequent accumulation of intracellular Ca^{2+} was not the cause of the elevated $[Ca^{2+}]_i$, because $^{45}Ca^{2+}$ efflux showed a slight but not statistically significant decrease in cells treated with NaCN. It is not known whether such a small decrease in Ca^{2+} efflux could contribute to the entire increase in $[Ca^{2+}]_i$ caused by cyanide. However, it seems unlikely because if a reduction of Ca^{2+} efflux was the cause, the cyanide-induced increase in $[Ca^{2+}]_i$ should be observed even in the absence of external Ca^{2+}. Such was not the case here. Meanwhile, this may exclude the possibility that Ca^{2+}-ATPase is affected by the cyanide-induced reduction of ATP levels in the cells [measured as a 32% reduction after a 10-min exposure (Kiang *et al.*, 1991)]. The fact that La^{3+} and Cd^{2+} (Ca^{2+} influx blockers) both blocked the cyanide-induced increase in $[Ca^{2+}]_i$ reinforces the view that cyanide stimulates Ca^{2+} influx.

Five observations suggest that the $[Ca^{2+}]_i$ increase is mediated by Na^+/Ca^{2+} exchange systems. First, the cyanide-

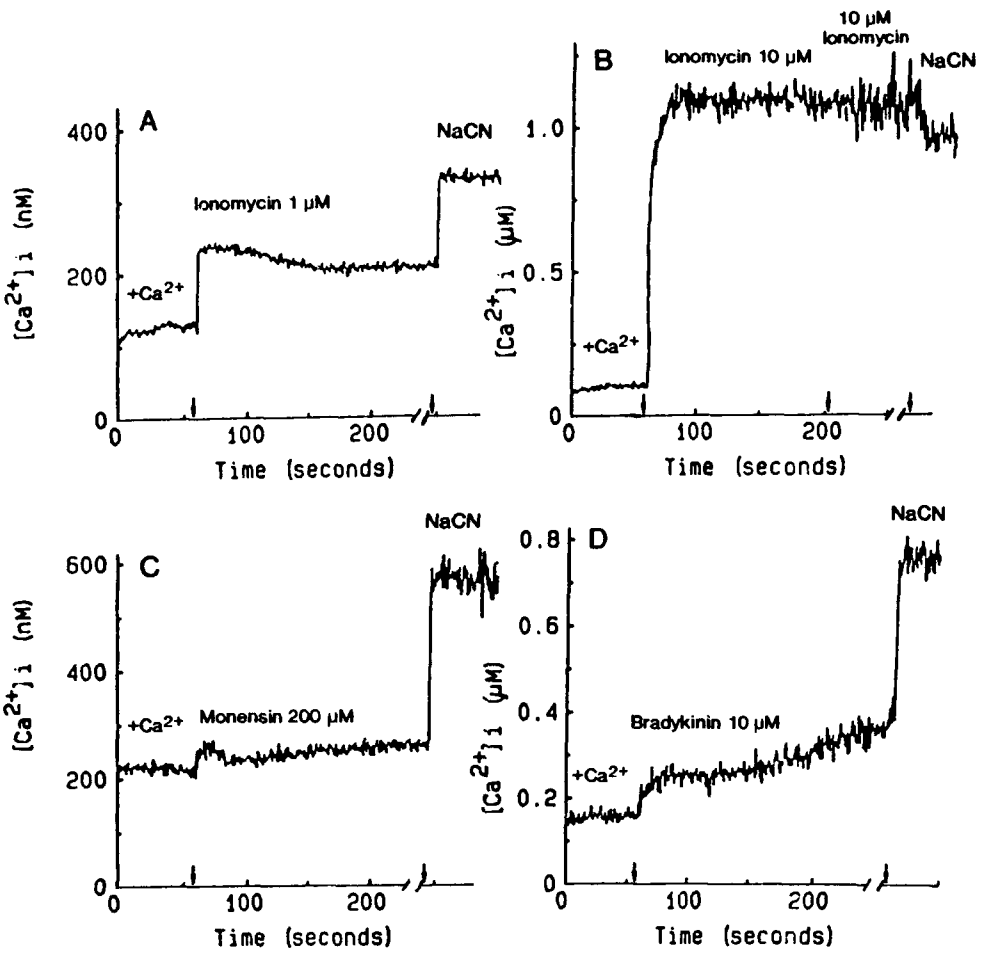

FIG. 7. Depletion of only the ionomycin-sensitive Ca^{2+} pools affects the increase in $[Ca^{2+}]_i$ induced by NaCN. Cells incubated in buffer containing 1.6 mM Ca^{2+} ($+Ca^{2+}$) were pretreated with (A) 1 μM ionomycin, (B) 10 μM ionomycin, (C) 200 μM monensin, or (D) 10 μM bradykinin prior to NaCN exposure (10 mM, 10 min). The break on the x axes represents the time interval for NaCN treatment.

induced increase in $[Ca^{2+}]_i$ was inhibited by La^{3+} and Cd^{2+}. Although La^{3+} and Cd^{2+} are effective inorganic Ca^{2+} channel blockers, they are known inhibitors of the Na^+/Ca^{2+} exchanger (Hagiwara, 1983; Trosper and Philipson, 1983). Second-messenger-operated Ca^{2+} channels are not affected by cyanide based on our results that cyanide decreased $InsP_3$. Second, cyanide caused an increase in $[Na^+]_i$ of 263 ± 16%. This increase in $[Na^+]_i$ was different from observations with hepatocytes. Kawanishi *et al.* (1991) reported that treatment with 2.5 mM KCN plus 0.5 mM iodoacetic acid increased $[Na^+]_i$ rapidly without changing $[Ca^{2+}]_i$. We found significant increases in both of these ions in A-431 cells treated with 2.5 mM NaCN ($[Ca^{2+}]_i$ increased 70 ± 5% and $[Na^+]_i$ increased 67 ± 4%). The differences between our data and theirs may be because of the different cells used and/or their combination of cyanide with iodoacetic acid. Third, the NaCN-induced increase in $[Ca^{2+}]_i$ depended on external $[Na^+]$. Fourth, changing the Na^+ gradient across the cell membrane by pretreatment with ouabain or veratridine, or removal of external Na^+, changed the cyanide-induced $[Ca^{2+}]_i$ increase, suggesting that the Na^+ entry is necessary. A role for extracellular Na^+ is indicated by several of our observations. Cyanide increased $[Na^+]_i$ only in the presence of extracellular Na^+. Moreover, pretreatment with tetrodotoxin completely inhibited the cyanide effect, indicating that Na^+ entry through tetrodotoxin-sensitive Na^+ channels plays a critical role. Schemes by which a cyanide-induced reduction of ATP decreased Na^+/K^+ ATPase activity leading to accumulation of intracellular Na^+ (Stallcup, 1986) do not reflect our findings. Fifth, amiloride and dichlorobenzamil, which block the Na^+/Ca^{2+} exchanger and Na^+ channels, completely blocked the cyanide effect.

It appears that the cyanide-induced influx of Ca^{2+} triggered mobilization of Ca^{2+} from intracellular Ca^{2+} pools because the Ca^{2+} mobilization blocker, TMB-8, diminished the cyanide response. This finding was supported by the data obtained from cells treated with ryanodine (an $InsP_3$-insensitive inhibitor of Ca^{2+}-induced Ca^{2+} mobilization) or

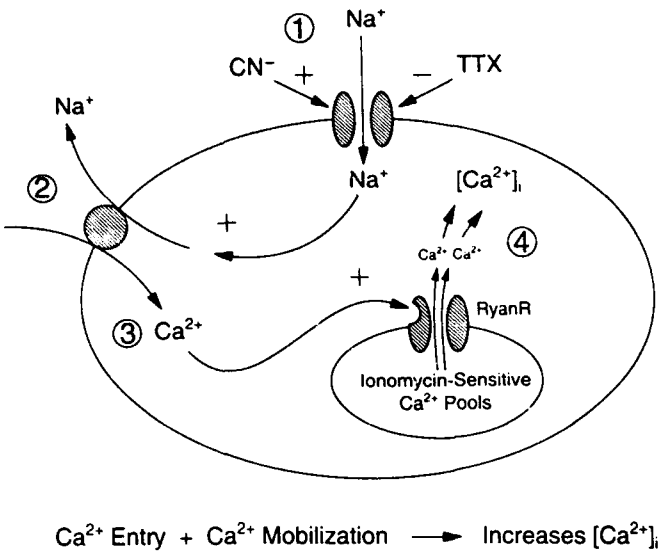

FIG. 8. Proposed mechanism of cyanide-induced $[Ca^{2+}]_i$ increase. Cyanide (CN−) activates tetrodotoxin (TTX)-sensitive Na^+ channels to increase $[Na^+]_i$ (step 1), which activates the reversed mode of the Na^+/Ca^{2+} exchanger (step 2), thereby increasing $[Ca^{2+}]_i$ (step 3). The increase in $[Ca^{2+}]_i$ subsequently mobilizes Ca^{2+} into the cytoplasm from ionomycin-sensitive Ca^{2+} pools through ryanodine receptor channels (step 4). RyanR, ryanodine receptor; +, stimulated activities; −, inhibited activities.

ruthenium red (an inhibitor of ryanodine receptor channels; Tsien, 1990). The Ca^{2+} pool that responds to the cyanide-induced Ca^{2+} influx was sensitive to ionomycin but not to bradykinin and monensin. This conclusion is based on three lines of evidence. (1) Data obtained from ryanodine and ruthenium red experiments indicate that $InsP_3$-sensitive Ca^{2+} pools are not involved in the cyanide response. (2) U-73122, an inhibitor of $InsP_3$ production (Kiang and McClain, 1993), did not inhibit the cyanide-induced increase in $[Ca^{2+}]_i$, but did inhibit the Ca^{2+} mobilization mediated by the bradykinin-induced increase in $InsP_3$. We also found that treatment with cyanide decreased the level of $InsP_3$. The finding of the cyanide-induced inhibition in $InsP_3$ is consistent with that observed in hepatocytes (Kawanishi *et al.*, 1991). (3) The ionomycin-sensitive Ca^{2+} pool was depleted by 1 μM ionomycin. In the presence of external Ca^{2+}, the increase in $[Ca^{2+}]_i$ induced by cyanide in cells pretreated with ionomycin (1 μM) was similar to that observed in cells pretreated with TMB-8 or ryanodine. When cells were saturated with Ca^{2+} by 10 μM ionomycin, NaCN caused no more changes in $[Ca^{2+}]_i$. Monensin or bradykinin had no effect on the increase in $[Ca^{2+}]_i$ stimulated by NaCN.

A proposed model by which cyanide increases cytosolic-free Ca^{2+} in A-431 cells is depicted in Fig. 8. Although the technique used in the present study could not determine the exact onset times of Na^+ and Ca^{2+} increases in the cytosol, our data seem to suggest that cyanide stimulates Na^+ entry through tetrodotoxin-sensitive Na^+ channels (step 1). The Na^+ entry stimulates the reverse mode of the Na^+/Ca^{2+} exchanger (step 2), resulting in an increased entry of Ca^{2+} (step 3). This Ca^{2+} then stimulates Ca^{2+} mobilization from ionomycin-sensitive Ca^{2+} pools containing ryanodine receptor channels (step 4). These pools cannot be depleted by either monensin or bradykinin. More studies are needed to understand how cyanide stimulates Na^+ channels and how Ca^{2+} entry stimulates ryanodine receptor channels to release Ca^{2+} from an ionomycin-sensitive pool. However, the mechanism for the $[Ca^{2+}]_i$ response to cyanide is similar to the heat shock-induced increase in $[Ca^{2+}]_i$ in A-431 cells (Kiang *et al.*, 1992) and the reperfusion-induced increase in $[Ca^{2+}]_i$ in rat heart cells (Lemasters *et al.*, 1993).

Other studies show that the large increase in $[Ca^{2+}]_i$ is a common pathway leading to cell death (Farber, 1981; Orrenius *et al.*, 1989; Trump and Berezesky, 1992; Geeraerts *et al.*, 1991). It is possible that this increase in $[Ca^{2+}]_i$ caused by cyanide is related to cyanide toxicity. Understanding the underlying mechanism of the cyanide-induced increase in $[Ca^{2+}]_i$ provides an opportunity to design therapeutic agents which can inhibit $[Ca^{2+}]_i$ increases caused by cyanide, thereby improving cell viabilities after a long exposure to cyanide.

In summary, cyanide caused increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ that depended on extracellular Na^+. The increase in $[Ca^{2+}]_i$ was inhibited by removal of external Ca^{2+} or by treatment with Cd^{2+}, La^{3+}, amiloride, or dichlorobenzamil. Cyanide also inhibited $InsP_3$. The data suggest that the increase in $[Na^+]_i$ was due to activation of tetrodotoxin-sensitive Na^+ channels, and that the increase in $[Ca^{2+}]_i$ was mediated by a Na^+/Ca^{2+} exchange system. This entry of Ca^{2+} stimulated Ca^{2+} release from an ionomycin-sensitive intracellular Ca^{2+} pool containing ryanodine receptor channels.

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